Pristopi za ocenjevanje endokrine aktivnosti: od mehanističnih bioloških testov do poti neželenih izidov

Endocrine activity assessment approaches: From mechanistic bioassays to adverse outcome pathways

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Ključne besede:
kemični motilci endokrinega sistema, visokozmogljivo presejanje, poti neželenih izidov, ocena okoljskega tveganja

Key words:
Endocrine-disrupting compounds; High-throughput screening; Adverse outcome pathways; Environmental risk assessment

Članek prispel / Received
4. 11. 2025
Članek sprejet / Accepted
12. 11. 2025

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Izvleček

Namen: *Predstaviti celovit pregled sodobnih pristopov za odkrivanje in karakterizacijo endokrine aktivnosti kemikalij ter poudariti povezavo med mehanističnimi* in vitro *testi,* in vivo *modeli in regulatornimi okviri.*
Metode: *Primerjane so bile novejše raziskave na področju visokozmogljivega presejanja (HTS), validiranih OECD preskusnih smernic in efektivno zasnovanih bioloških testov. Pregled zajema reprezentativne teste za estrogene, androgene, ščitnične in steroidogene poti ter ocenjuje njihovo dopolnjevanje znotraj okvira Poti neželenih izidov (AOP). Posebna pozornost je bila namenjena integraciji podatkov, upoštevanju biološke uporabnosti in regulatornemu sprejemanju testov v okviru uredb REACH ter ameriškega programa* Endocrine Disruptor Screening Program (EDSP).
Rezultati: *Mehanistični testi, kot so*

Abstract

Purpose: *To provide an integrated overview of current approaches for detecting and characterizing endocrine activity in chemicals by emphasizing the relationship between mechanistic* in vitro *assays,* in vivo *models, and regulatory frameworks.*
Methods: *Recent developments in high-throughput screening (HTS) platforms, validated Organisation for Economic Co-operation and Development (OECD) test guidelines, and effect-based bioassays were critically compared. The review covers representative assays for estrogenic, androgenic, thyroid, and steroidogenic pathways and evaluates the complementarity within adverse outcome pathway (AOP) frameworks. Attention was given to data integration, bioavailability considerations, and regulatory adoption under the European Union Registration, Evaluation, Authorization,*

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YES, E-Screen, HeLa-9903 in H295R, omogočajo hitro, občutljivo in stroškovno učinkovito presejanje receptorsko posredovanih in steroidogenih učinkov, medtem ko in vivo sistemi, kot so indukcija vitelogenina, reprodukcijski testi pri vodnih bolhah, metamorfoza pri dvoživkah in testi na zebricah, zagotavljajo potrditev učinkov na ravni organizma. Integracija obeh pristopov v okviru AOP ter kvantitativne ekstrapolacije in vitro-in vivo (QIVIVE) *povečujejo napovedno zanesljivost testov. Kljub napredku v standardizaciji pa ostajajo izzivi, povezani z učinki metabolitov, med-laboratorijsko variabilnostjo in usklajenimi določitvami mejnih učinkov.*

Zaključek: *Znanost o preskušanju endokrine aktivnosti se usmerja v mehanistično bazirane pristope, ki zmanjšujejo porabo živali za testiranje in združujejo visokozmogljivo presejanje, računalniško modeliranje in efektivno zasnovano spremljanje. Usklajeni kriteriji za validacije in standardizirani izidi bodo dodatno okrepili regulatorno sprejemanje testov ter omogočili učinkovitejšo identifikacijo snovi z endokrino aktivnostjo v širšem kontekstu zdravja ljudi in okolja.*

and Restriction of Chemicals (REACH) and the US Environmental Protection Agency (EPA) Endocrine Disruptor Screening Program.

Results: *Mechanistic assays, such as YES, E-Screen, HeLa-9903, and H295R, provide rapid, sensitive, and cost-effective screening for receptor-mediated and steroidogenic effects. In contrast,* in vivo *systems, including vitellogenin induction,* Daphnia *reproduction, amphibian metamorphosis, and zebrafish assays, yield organism-level confirmation. Integrating both tiers within AOP and quantitative* in vitro-*to*-in vivo *extrapolation (QIVIVE) frameworks increases predictive reliability. Despite advances in standardization, challenges remain concerning metabolic competence, inter-laboratory variability, and harmonized effect thresholds.*

Conclusion: *Endocrine testing science is transitioning toward mechanistically anchored, animal-reduced strategies that combine HTS, computational modeling, and effect-based monitoring. Harmonized validation criteria and performance standards will further enhance regulatory acceptance and facilitate the efficient identification of substances with endocrine activity across both human health and environmental contexts.*

INTRODUCTION

Substances with endocrine effects, such as pesticides, plasticizers, pharmaceuticals, and personal care products, can be dispersed through wastewater effluents and environmental compartments, resulting in continuous, low-level exposures across ecosystems and the human population (1). Determining the effects of such compounds on the endocrine system is a complex process. The most comprehensive set of tests for identifying potential endocrine-disrupting chemicals (EDCs) is part of the United States Environmental Protection Agency (US EPA) screening program, with total testing costs exceeding one million USD per compound (2). *In vitro* and *in silico* high-throughput screening (HTS) procedures have become predominant in recent years due to the vast number of substances on the market because HTS procedures enable the collection of data for many compounds at a substantially lower cost (3). It is impossible to determine the influence of such a large array of compounds on all possible cellular receptors while considering the complexity and interconnections of biochemical processes in the body (4). The principle of these assays is to only identify the key molecular targets and reaction mechanisms through which endocrine disruptors can act, then to quantify the effects on these targets. The primary objective of all high-throughput approaches is to identify candidate substances for further testing in smaller, yet more reliable, assay systems, including *in vivo* studies (5). Therefore, the current article focuses on the detection and characterization of endocrine activity as part of the path to identifying EDCs.

ORGANISATION FOR ECONOMIC CO-OPERATION AND DEVELOPMENT FRAMEWORK

Each active substance is tested using a series of assays that address acute, sub-chronic, and chronic exposures in accordance with internationally accepted guidelines (6). Compounds are categorized into five levels based on the data obtained, as shown in Figure 1 (4). Existing data and non-testing information are evaluated in Level 1, including physicochemical properties, results from standard toxicity tests, *in silico* toxicity predictions, and pharmacokinetic modeling. Level 2 builds upon this foundation with *in vitro* assays that generate mechanistic data on endocrine activity in mammals and other species, such as binding affinity to estrogen (ER) or androgen (AR) receptors, activation of ER and AR, *in vitro* steroidogenesis, and activation of thyroid or retinoid receptors. These assays help identify molecular initiating and key events at the cellular level. Level 3 introduces first *in vivo* mechanistic assays which bridge molecular mechanisms with organismal responses. Representative tests include the uterotrophic and Hershberger assays, amphibian metamorphosis, short-term fish reproduction assays, and short-term endocrine activity tests in *Daphnia*. Together, Levels 2 and 3 form the mechanistic evidence tier, describing how a substance interacts with endocrine pathways and how these interactions manifest in short-term biological responses. Level 4 comprises more complex *in vivo* studies that provide apical data, capturing adverse effects mediated through endocrine-relevant targets. These include 28- and 90-d repeated-dose toxicity studies, one-generation reproductive toxicity studies, developmental and pubertal assays, and

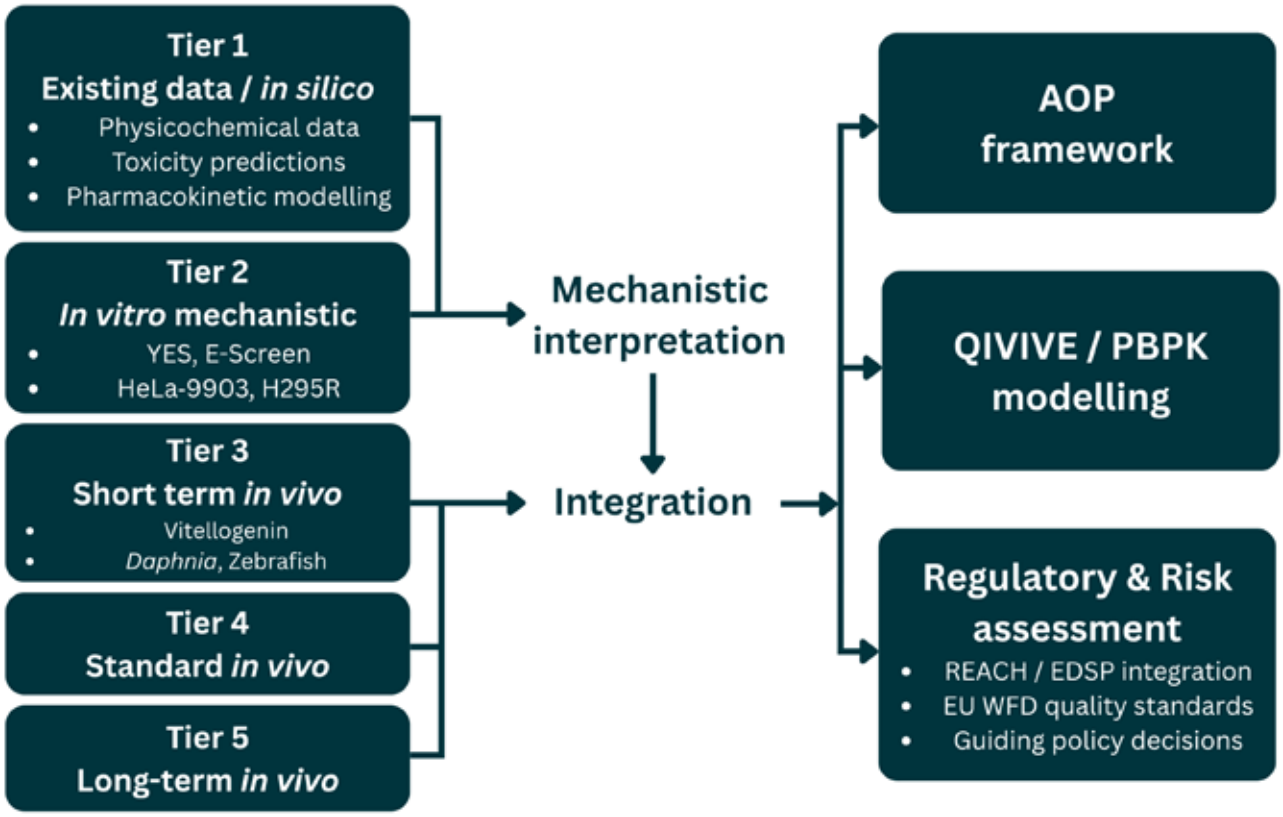

Figure 1. *Integrated endocrine testing and risk assessment framework. The tiered testing approach integrates into planning of adverse outcome pathway (AOP) frameworks, modelling of quantitative in vitro-to-in vivo extrapolation (QIVIVE), and physiologically based kinetic/pharmacokinetic (PBPK) models and ultimately regulatory requirements and risk assessments.*

chronic reproductive studies in fish and mammals. Level 5 represents the highest tier, encompassing long-term apical tests that integrate effects across multiple life-cycle stages and generations, such as the extended one- or two-generation reproductive toxicity studies and multi-generational fish or *Daphnia* tests.

Thus, the hierarchy connects mechanistic data (Levels 2–3), which explain how a substance interacts with the endocrine system, to apical data (Levels 4–5), which demonstrate the biological consequences these interactions cause at the organism and population levels. This finding is why comprehensive and robust Level 1 data are mandatory. Levels 2 and 3 are required if other studies raise suspicion. Level 4 and 5 studies provide increasing confidence in detecting endocrine effects. Level 5 is only required in specific ecotoxicological cases (6).

MECHANISTIC BASIS OF ENDOCRINE ACTIVITY

The primary site of action in the estrogenic hormonal system involves the interaction of endocrine-active substances with ERs, acting as either agonists or antagonists. Several molecular events are involved, as follows: ligand binding; receptor dimerization; nuclear translocation; formation of the transcriptional complex with cofactors; DNA binding; transcription; translation; post-translational modification; and cellular response. In addition to receptor-mediated signaling, several endocrine-disrupting compounds have been shown to affect DNA integrity and gene expression through oxidative stress, altered DNA methylation, and chromatin remodeling mechanisms (7). Because individual assays generally measure only one or a few of these processes, advanced multi-assay models, such as those in the US EPA Tox21 program, apply up to 18 complementary tests. Statistical integration of these results allows classification of a compound as an estrogenic agonist, antagonist, active through non-estrogenic pathways, or inactive (8). Optimization studies on approximately 1800 compounds have shown that comparable accuracy can be achieved with only 4 key assays, each targeting a distinct step in ER signaling (9). Developing models for evaluating effects on the androgenic system is even more complex due to the limited availability of reference compounds and technical challenges. Many chemicals act as AR antagonists and can induce cytotoxicity *in vitro*, producing false-positive results. Therefore, simultaneous cytotoxicity evaluation and co-exposure with known agonists are used to confirm antagonism and reduce false-positive results (10). Steroidogenesis is another critical endpoint. *In vitro* steroidogenesis assays and aromatase activity measurements reveal broader endocrine interference across axes, which helps to capture effects that single-receptor tests might miss (11). In addition to direct receptor binding, endocrine-active substances may interfere with intracellular signaling cascades, hormone transport, or metabolism. Crosstalk among nuclear receptor families, such as peroxisome proliferator-activated receptors (PPARs), aryl hydrocarbon receptor (AhR), and retinoid X receptor (RXR), can modulate endocrine responses without classic ER, AR, or thyroid hormone receptor (TR) activation. These non-receptor mechanisms expand the conceptual boundary of endocrine activity that can lead to endocrine disruption and should be incorporated into broader adverse outcome pathway (AOP) frameworks and HTS platforms to improve detection of indirect hormonal effects. This interconnection of different mechanisms is also the reason AOPs are a necessary framework for accurate effect determination.

Yeast Estrogen Screen

The yeast estrogen screen (YES) uses genetically modified *Saccharomyces cerevisiae* cells that express human ERα and contain an estrogen-responsive element linked to a reporter gene (typically *lacZ*). Upon activation, β-galactosidase is produced, converting a chromogenic substrate from red-to-yellow for spectrophotometric measurement. Modern versions of the YES use bioluminescent reporters (12). The assay detects agonism and competitive antagonism but is limited to ER-mediated activity, which is only a single component of the endocrine system. The YES is simple, low-cost, fast (< 24 h), sensitive (ng/L for estrogens), and robust to matrix effects. Multiple strains and commercial kits are available (13, 14).

E-screen

E-screen uses human MCF-7 cells to measure estrogen-induced proliferation. This cell line expresses ERα (and minor ERβ) and contains aromatase and 5α-reductase, enabling the conversion of androgens-to-estrogens. E-screen is generally more sensitive compared to yeast systems and detects agonists and antagonists. However, non-estrogenic mitogens can cause non-specific responses, which causes false-positive results. In addition, the assay can last up to 7 d even though automation and parallel analysis of many samples are feasible. Accelerated readouts include estrogen-responsive genes, such as *pS2* mRNA, which is detectable within hours (15, 16). Compounds may test negative in this assay yet show activity in more complex *in vivo* assays [e.g., vitellogenin (VTG)] (17).

hERα-HeLa-9903

The hERα-HeLa-9903 transcriptional activation assay, which is part of US EPA screening, measures ERα-driven luciferase expression in engineered HeLa cells (18). Bioluminescence correlates with receptor activation with estradiol serving as the positive control. The assay identifies agonists and provides mechanistic insight but cannot predict whole-organism outcomes influenced by metabolism, endocrine feedback, receptor crosstalk, and pharmacokinetic processes. In addition, some compounds may cause false-positive results at high concentrations by directly activating luciferase without receptor binding (19). Standardized commercial kits improve inter-laboratory comparability (20, 21).

VTG

VTG is an egg-yolk precursor synthesized in the liver of adult females in most oviparous species. VTG is induced by estradiol. Induction of VTG in males serves as a sensitive biomarker of estrogen exposure (22). VTG is commonly quantified in fish, such as *Pimephales promelas*, as mRNA (indicating a rapid response) or protein (indicating long-term exposure) using an enzyme-linked immunosorbent assay (ELISA) or liquid chromatography-mass spectrometry (LC-MS). ELISA is a practical and sensitive method, although results can vary depending on the antibodies and standards used. Standardized kits recommended by the guideline of the Organisation for Economic Co-operation and Development (OECD) number 229 help mitigate this variability, although only partially (23, 24). Non-lethal sampling from epidermal mucus is also possible (25).

Daphnia

Daphnia magna, a small planktonic crustacean, reproduces by cyclical parthenogenesis, which allows for rapid multigenerational observation. Chronic tests monitor survival, size, morphology, fecundity, and sex ratio under controlled conditions. The OECD test number 211 (21-d reproduction test with at least 10 individuals) is used and considered the gold standard for assessing chronic reproductive toxicity. However, *Daphnia* lacks classical vertebrate steroid receptors, meaning the endocrine signaling pathways differ substantially from fish or mammals and are therefore not suitable for identifying specific endocrine molecular mechanisms (26, 27).

Zebrafish

Zebrafish (*Danio rerio)* offer a human-relevant *in vivo* system due to transparent embryos, rapid organogenesis, high fecundity, and approximately 70% genome homology with humans, which is why zebrafish partly bridge the gap between mechanistic *in vitro* assays and vertebrate testing (28). Experimental endpoints include reproductive, developmental, histologic, and behavioral measures, such as swimming activity (29). A variant of the zebrafish system, the EASZY assay, utilizes cyp19a1b-GFP transgenic embryos and is currently under evaluation by the OECD as a method for detecting endocrine-active substances. The main limitations of the zebrafish system lie in the fact that the interpretation of results is influenced by developmental stage, duration of exposure, and non-specific toxicity, while the tests remain comparatively resource-intensive and require careful standardization (30, 31).

Thyroid and Steroidogenic Pathways

The thyroid axis regulates metabolism, growth, and neurodevelopment through thyroxine (T4) and

triiodothyronine (T3). Endocrine-active chemicals disrupt this axis by inhibiting thyroid peroxidase (TPO), altering sodium-iodide symporter (NIS) function and iodide uptake, and competing for transport proteins, such as transthyretin, modulating deiodinases (D1-D3), or interacting with nuclear thyroid hormone receptors (TRα/β). Regulatory test systems capture these modes of action at multiple biological levels. The amphibian metamorphosis assay [OECD TG 231] (32) quantifies thyroid-dependent developmental timing in *Xenopus laevis* tadpoles using hind limb growth and stage progression as sensitive endpoints. Fish-based assays, such as the fish short-term reproduction assay [OECD TG 229] can include thyroid-relevant biomarkers with reproductive measures (33). Similarly, steroidogenesis is closely linked to thyroid status and is a major route of interactions with endocrine activity. The H295R steroidogenesis assay [OECD TG 456] (34) uses human adrenocortical carcinoma cells to quantify altered estradiol and testosterone production after chemical exposure, while targeted enzyme assays (aromatase/CYP19A1 and 17β-HSD) identify specific nodes affected (35). Together, these assays enable mechanistic weight-of-evidence determinations that support translation to organism-level outcomes when integrated with toxicokinetic data. However, interspecies variability in thyroid regulation and limited metabolic capacity in steroidogenic models underscore the need for complementary endpoints and integrative interpretation within the AOP and extrapolation frameworks to ensure reliable translation from mechanistic data to organism-level effects.

MIXTURES, ENVIRONMENTAL RELEVANCE, AND BIOAVAILABILITY

Environmental exposures rarely involve single chemicals. Instead, organisms encounter mixtures with diverse modes of action at the sub-ng/L-to-µg/L levels (36). Mixture effects can be additive (concentration addition), independent (response addition), or antagonistic/synergistic and are strongly influenced by bioavailability and metabolism. Classical *in vitro* tests typically use nominal concentrations and lack metabolic competence, which may misrepresent internal dosimetry and active metabolites (37). Bridging assays with exposure science improves realism. Specifically, passive samplers (e.g., silicone sheets and disks) and polar organic chemical integrative sampler (POCIS) devices provide time-integrated extracts that can be assayed directly (effect-based monitoring). Effect results can be expressed as equivalency metrics (e.g., estradiol equivalents [E2eq] for ER activity or dihydrotestosterone equivalents [DHTeq] for AR), allowing comparison to effect-based trigger values proposed for water quality assessment (38). Coupling bioassays with toxicokinetic modeling and quantitative structure-property relationship-based bioavailability estimates refines mixture interpretation, while fractionation (HPLC or SPE) can deconvolute mixture drivers. Ultimately, mixture-aware assessment benefits from combining effect-directed analysis with targeted high-resolution mass spectrometry (HRMS) to link bioactivity to toxicologically active substances (39).

DATA INTEGRATION AND ADVERSE OUTCOME PATHWAYS

Large-scale programs, such as Tox21 and ToxCast, generate HTS data across hundreds of receptors and enzymes relevant to endocrine biology. To translate these data into decisions, the AOP framework links molecular initiating events to key events and adverse outcomes at the organism or population level (40). Quantitative AOPs (qAOPs) further incorporate dose-response and time-to-event information, enabling predictions under realistic exposure scenarios. Integration pipelines combine HTS potency metrics (e.g., AC_{50} and area under the curve), cytotoxicity filtering, and chemotype alerts with quantitative *in vitro*-to-*in vivo* extrapolation (QIVIVE) and physiologically based kinetic/pharmacokinetic (PBK/PBPK) models to estimate external doses that produce equivalent internal target effects (41). Machine learning models trained on curated HTS and apical datasets support read-across and prioritization, while flagging uncertainty (42). However, these computational methods do not replace validated test

Table 1. *Overview of Representative Assays for Assessing Endocrine Activity*

Assay / System	Biological Level	Primary Endpoint(s)	Typical Duration	OECD Test Guideline	Approximate Cost / Throughput	Notes / Strengths
YES (Yeast Estrogen Screen)	*In vitro* (yeast, estrogen receptor - ERα)	Estrogen receptor activation (agonism/ antagonism)	< 1 day	-	Low / High	Simple, rapid, inexpensive; limited to ER-mediated effects
E-Screen (MCF-7)	*In vitro* (human cells)	Estrogen-dependent proliferation	3-7 days	-	Moderate / Moderate	Sensitive; detects genomic & non-genomic activity; some nonspecificity
hERα-HeLa-9903	*In vitro* (human cells)	ERα transcriptional activation (luciferase reporter)	≤ 1 day	TG 455	Moderate / High	Standardized; detects agonists/antagonists; no metabolism
H295R Steroidogenesis	*In vitro* (adrenocortical cells)	Estradiol/testosterone synthesis alteration	1-2 days	TG 456	Moderate / Moderate	Mechanistic assay targeting steroid biosynthesis
Vitellogenin (VTG)	*In vivo* (fish)	Plasma/hepatic VTG induction	7-21 days	TG 229 / 230	High / Low	Sensitive; biologically relevant; species dependent
Daphnia Reproduction Test	*In vivo* (crustacean)	Survival, fecundity, sex ratio	21 days	TG 211	Moderate / Low	Chronic ecotoxicological relevance
Amphibian Metamorphosis Assay	*In vivo* (amphibian)	Developmental stage, hind-limb growth	21 days	TG 231	High / Low	Specific for thyroid activity; regulatory acceptance
Zebrafish (EASZY, etc.)	*In vivo (*fish)	Transgenic ER-responsive gene expression, reproduction	7-30 days	TG 234 / in validation	High / Moderate	High-content developmental endpoints; suited for screening

guidelines but enhance prioritization and reduce animal use by focusing *in vivo* testing on the most plausible risks.

COMPARATIVE OVERVIEW OF METHODS

The scope of endocrine testing ranges from mechanistic precision to ecologic relevance, as presented in Table 1. *In vitro* systems, such as YES, HeLa-9903, and H295R, provide rapid, mechanistically specific information, while *in vivo* assays (VTG induction, *Daphnia* 21-d reproduction, zebrafish embryo tests, and amphibian metamorphosis) capture whole-organism adversity. Integrating both levels in a stepwise or evidence-based sequence enables efficient screening, while maintaining biological relevance. Validation and regulatory acceptance vary, as follows ER/AR transcriptional activation assays align with OECD TG 455 principles; VTG is a well-established biomarker in fish population models; and OECD TG 211 (*Daphnia*) and TG 231 (amphibian metamorphosis) are widely recognized for chronic and thyroid-specific endpoints. Cost-time trade-offs favor a tiered strategy, which first deploys HTS to map mechanistic space and derive potency rankings, followed by targeted *in vivo* tests to confirm adversity at relevant life stages. Effect-based monitoring, when feasible, is used to connect laboratory potency with field exposures. Tabulating sensitivity, specificity, typical runtime, and indicative costs per assay family can further support study design and regulatory submissions.

FUTURE PERSPECTIVES

Endocrine toxicology is shifting toward animal-reduced, mechanism-driven assessment. Emerging tools include 3D organoids and microphysiologic systems (organ-on-chip) that reproduce human tissue architecture and hormone feedback loops, single-cell and spatial omics that reveal hormone-responsive cell states, and label-free biosensors that enable continuous ER/AR/TR signaling readouts (43). HRMS (untargeted and suspect screening) accelerates effect-directed analysis, while computational docking and deep learning improve virtual screening for receptor binding and enzyme inhibition (44). Standardization efforts by the OECD and the International Organization for Standardization (ISO) with open data infrastructures, such as the CompTox Chemicals Dashboard, are crucial for interoperability. Future priorities include establishing performance standards for novel models, routinely embedding toxicokinetics/QIVIVE, and harmonizing effect-based trigger values across jurisdictions to enable bioassays to inform regulatory thresholds with conventional chemical monitoring (45).

REGULATORY AND RISK ASSESSMENT IMPLICATIONS

Regulatory decision-making increasingly integrates mechanistic and apical evidence within transparent frameworks. REACH and the Biocidal Products Regulation in the European Union use criteria for identifying endocrine disruptors, which are supported by guidance from the European Chemicals Agency (ECHA)/European Food Safety Authority (EFSA). Annex XV dossiers and weight-of-evidence approaches encourage combining *in vitro* mechanistic data (e.g., ER/AR transactivation assays and H295R) with *in vivo* adversity data (e.g., TG 231 and TG 211) and toxicokinetic information (46). The Endocrine Disruptor Screening Program in the US leverages ToxCast/Tox21 data for Tier 1 screening and uses Tier 2 tests for confirmation (47). Water-quality management bodies increasingly use effect-based monitoring with bioassays (ER-CALUX/YES, AR-CALUX, and H295R) with targeted chemical analysis, guided by emerging effect-based trigger values in technical reports under the EU Water Framework Directive (48). The adoption of the AOP framework supports structured evidence evaluation across jurisdictions, while QIVIVE/PBPK models help translate *in vitro* potency into external exposure benchmarks, such as derived no-effect levels or environmental quality standards. Citing specific OECD Test Guidelines (e.g., TG 229, 231, 234, and 456) and providing links to official documents for submissions strengthens traceability and facilitates regulatory review. Despite major progress, several limitations persist. Many *in vitro* systems lack metabolic activation, potentially underestimating the activity of biotransformation-dependent disruptors. Inter-laboratory variability, differences in reference materials, and incomplete data integration frameworks still constrain cross-study comparability. Furthermore, effect-based trigger values and potency thresholds for bioassays remain inconsistently defined across regions (49). Future harmonization of assay validation, the inclusion of metabolic effects, and the establishment of standardized effect thresholds are essential to achieve regulatory convergence and enhance predictive power.

CONCLUSIONS

The described assays together form a comprehensive toolkit for identifying and characterizing endocrine-active substances. A robust assessment of endocrine activity requires the detection of receptor-level interactions, as well as the integration of toxicokinetic, organismal biology, and ecologic relevance. Combining *in vitro* mechanistic assays, *in vivo* functional studies, exposure science, and computational modeling within the AOP framework provides an efficient, predictive, and ethically sustainable path forward.

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